

Photosensitized NADH formation system with multilayer TiO₂ film†Takashi Sagawa,^{*a} Ryota Sueyoshi,^b Mikako Kawaguchi,^a Mayu Kudo,^a Hirotaka Ihara^b and Katsutoshi Ohkubo^{*a}^a Institute of Advanced Energy, Kyoto University, Gokasho, Uji 611-0011, Japan.

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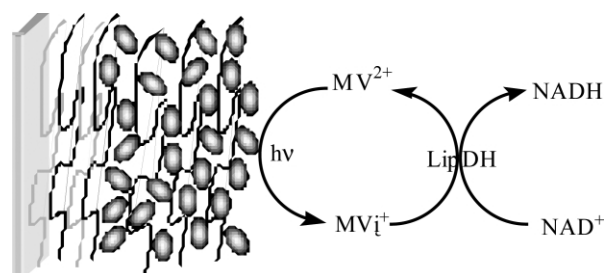
A TiO₂/polymer film on a quartz plate fabricated by a layer-by-layer method was employed for NADH production from NAD⁺ with lipoamide dehydrogenase and methyl viologen in Tris-HCl buffer on irradiation with UV light, and the ultra thin film prevented from enzyme deactivating effectively.

Layer-by-layer self-assembly, based on spontaneous ionic adsorption of oppositely charged materials from their solution can be a promising technique to fabricate thin films on solid supports.^{1–3} Recently, many groups have been interested in preparing thin films with nanoparticles of semiconductors and metals onto transparent matrixes by using the layer-by-layer method. In particular, several groups have reported the fabrication and photophysical characterization of layer-by-layer assembly of TiO₂ thin films embedded on a quartz slide.^{4–12} TiO₂ is an n-type semiconductor and a typical photocatalyst for environmental purification.¹³ However, little is known about photocatalytic reactions using such layer-by-layer assemblies of TiO₂ thin films. Currently, there are no reports of applications of some enzymatic reactions. Dihyronicotinamide adenine dinucleotide (NADH) plays an important role as a cofactor in various biological reactions. Several effective chemical systems for generation of NADH have been reported using polymer-modified electrodes, homogeneous metal complexes of Ru(bpy)₃²⁺ or Rh(bpy)₃³⁺, or dispersed semiconductor particles of TiO₂ as a photosensitizer in the presence of an electron mediator such as methyl viologen (MV²⁺) and/or using dehydrogenase, which catalyzes the reduction of NAD⁺.^{14–22} However, these systems are inefficient for practical use because they require expensive sacrificing reagents as electron donors such as ethylenediaminetetraacetic acid,¹⁴ glucose 6-sulfate,¹⁵ methanol,^{16,22} 2-mercaptoethanol,¹⁷ 2-propanol,¹⁸ formic acid,¹⁹ triethylamine,²⁰ or electricity²¹ itself. It is also difficult to recover and reuse the photosensitizers from the bulk because of their homogeneous or dispersed states. Recently, we reported the fabrication of TiO₂/polymer films on a quartz substrate by the layer-by-layer method with cationic poly(diallyldimethylammonium chloride) (PDDA) and anionic potassium poly(vinyl sulfate) (PVS).¹² The transparent ultra thin films showed photocatalytic activity for reduction of methylviologen (MV²⁺) when irradiated with UV light. These ultra thin films show no decrease in the photocatalytic activity over 10 cycles under Xe lamp light-illumination. We present here a more practical and sustainable system for production of NADH by using a TiO₂-layered film as a reusable photocatalyst, MV²⁺ as an efficiently mediating electron carrier, and lipoamide dehydrogenase (LipDH; E.C. 1.8.1.4)‡ as an enzyme (Scheme 1).

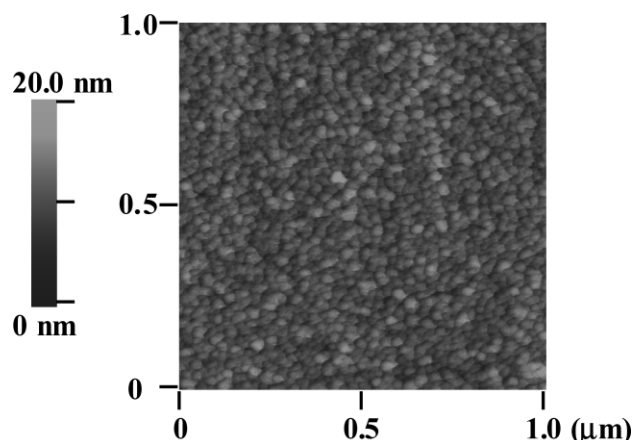
TiO₂ nanocrystallite was prepared as described by Hoffmann *et al.*²³ An X-ray diffraction pattern of the TiO₂ powder at room temperature indicates the dominant peak for anatase. The diameter of colloidal anatase in water (pH 7.0) was determined by dynamic light scattering. The average diameter was *ca.* 10 nm. Layer-by-layer self assembly of cationic TiO₂ nanoparticles between anionic PVS layers was performed as previously described.¹² 20 layers (one

side) of TiO₂ were immobilized onto a quartz plate of 30 × 9.0 × 1.0 mm³. The total weight of immobilised TiO₂ onto the quartz surface was 1.34 μg (both sides). Surface morphology was clearly seen by an atomic force microscopy (AFM) image of a 5-layered TiO₂ film on a substrate of quartz (Fig. 1). It shows the densely packed TiO₂ nanoparticles on the solid substrate. A height profile along a certain axis showed periodical top-valley patterns with depth of 0.5–7 nm and width of 20–50 nm. Though the average diameter of the colloidal anatase was 10 nm, the AFM image indicates much larger particles due to aggregation in the self-assembled films.

The photochemical system for generation of NADH was composed of an aqueous 0.2 mol dm⁻³ Tris-HCl buffer solution (pH 7.0) that included NAD⁺ (0.5 mmol dm⁻³), MV²⁺ (0.5 mmol dm⁻³), and LipDH (30 units) with a 20-layered TiO₂-immobilised quartz plate. Samples (3 cm³) consisting of all the components, were transferred to a quartz glass cuvette (10 × 10 × 40 mm³) and were purged of oxygen by argon flushings. The cuvette was equipped with a small magnetic stirrer bar and illumination was performed at room temperature equipped with a 500 W xenon lamp. Light was filtered through a λ > 325 nm cutoff filter. The production of NADH and MV⁺ was followed spectrophotometrically at 340 nm (ε 6.2 × 10³ M⁻¹ cm⁻¹ for NADH) and 605 nm (ε 1.24 × 10⁴ M⁻¹ cm⁻¹ for MV⁺), respectively. Photoirradiation of an aqueous solution of Tris-HCl buffer that includes the



Scheme 1

Fig. 1 Atomic force microscopic image of TiO₂ film onto a quartz.† Electronic supplementary information (ESI) available: experimental and spectroscopic data. See <http://www.rsc.org/suppdata/cc/b3/b315382g/>

photocatalyst of the TiO₂-immobilised quartz plate, MV²⁺, NAD⁺ and LipDH results in the formation of MV^{•+} (393 and 605 nm) and NADH (340 nm) as shown in Fig. 2. The rate of NADH formation was followed at 340 nm and corresponds to a quantum yield of Φ 0.033 in the case of illumination for 30 min. Exclusion of the enzyme LipDH or electron mediator MV²⁺ prevents the formation of NADH. Furthermore, exclusion of the buffer reagent of Tris-HCl also prevents the production of NADH and MV^{•+}. Addition of Tris to the aqueous solution that includes the TiO₂-immobilised quartz plate, MV²⁺, NAD⁺ and LipDH results in generation of MV^{•+} and concomitant formation of NADH. These results imply that Tris plays an important role not only as a buffer reagent but also a hole scavenger (*viz.* sacrificing reagent) for photoactivated TiO₂ semiconductor.

LipDH can recognise viologen as a reductant for NADH formation. However, little is known about the mechanisms in this photo-enzymatic reaction system. We examined kinetic analysis for the oxidation of MV^{•+} with LipDH as follows. After the 20 min photoirradiation of a mixture of a TiO₂-immobilised quartz plate, MV²⁺ (0.5 mmol dm⁻³), LipDH (2 units) in 0.2 mol dm⁻³ Tris-HCl buffer (pH 7.0) under Ar, 0.5 mmol dm⁻³ of NAD⁺ was added to the solution and were monitored as a function of time changes in the absorbance at 605 nm. The initial concentration of MV^{•+} was 148 μ mol dm⁻³ and the final concentration of NADH produced was 64 μ mol dm⁻³. This indicates that two equivalents of MV^{•+} are required for one NADH formation with LipDH. In other words, this reaction system is able to supply two electrons and a proton for one NADH generation. Second order rate constants were estimated from the above experiments and Michaelis-Menten analyses were performed. Kinetic parameters of V_{\max} and K_m^{-1} from Lineweaver-Burk plots are summarized in Table 1. In the case of TiO₂ film utilisation, both values of V_{\max} and K_m^{-1} were larger than the case of dispersed colloidal TiO₂ (the same amount as the film) utilisation. This result implies that excess contact of protein with the photocatalyst might reduce the enzyme activity. On the other hand, TiO₂/polymer film on quartz plate might suppress the deactivation of the enzyme from the photoactivated species efficiently in this photo-enzymatic reaction system. The TiO₂/

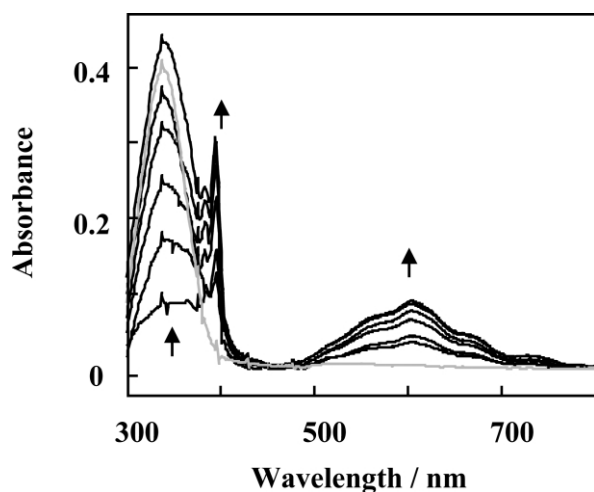


Fig. 2 MV^{•+} and NADH formation at time intervals of 5 min of illumination (black solid lines from 5 min to 30 min): 0.2 mol dm⁻³ Tris-HCl buffer at pH 7.0 containing a 20-layered TiO₂ film, MV²⁺ (0.5 mmol dm⁻³), NAD⁺ (0.5 mmol dm⁻³), and 30 units of LipDH. Exposure of the reaction mixture to the air resulted the disappearance of MV^{•+} (gray line).

Table 1 Kinetic parameters of V_{\max} and K_m^{-1} for oxidation of MV^{•+} with LipDH^a

Catalyst	$V_{\max}/$ $\mu\text{mol dm}^{-3} \text{ min}^{-1}$	$K_m^{-1}/$ $\mu\text{mol}^{-1} \text{ dm}^3$
TiO ₂ /polymer film	382	7.69×10^{-3}
Colloidal TiO ₂	138	3.92×10^{-3}

^a Enzyme reactions were done at the various concentration of MV^{•+} from 50 μ mol dm⁻³ to 120 μ mol dm⁻³ as described in the text.

polymer film is also able to be used repeatedly over 20 times without decomposition and subsequent removal of the film reveals similar results.¹² Many enzymatic reduction processes are dependent on the NADH cofactor. Therefore coupling of the photosensitized NADH regeneration cycles with secondary enzyme-catalyzed processes is expected to allow various synthetic applications.

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Notes and references

‡ LipDH, type III from porcine heart was purchased from Sigma-Aldrich Fine Chemicals.

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